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**Diabetes-induced alterations in urothelium function:
enhanced ATP release and nerve-evoked contractions in the streptozotocin
rat bladder**

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Short title: Altered urothelial function in diabetes

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Abstract

Up to 80% of patients with diabetes mellitus develop lower urinary tract complications, most commonly diabetic bladder dysfunction (DBD). The aim of this study was to investigate the impact of diabetes on the function of the inner bladder lining (urothelium). Bladder compliance and intraluminal release of urothelial mediators (ATP and ACh) in response to distension was investigated in whole bladders isolated from 2 and 12-week streptozotocin (STZ)-diabetic rats. Intact and urothelium-denuded bladder strips were used to assess the influence of the urothelium on bladder contractility. Intraluminal ATP release was significantly enhanced at 2 weeks of diabetes, although not at 12 weeks. In contrast, intraluminal ACh release was unaltered by diabetes. Bladder compliance was also significantly enhanced at both 2 and 12 weeks, of diabetes, with greatly reduced intravesical pressures in response to distension. Nerve-evoked contractions of bladder strips were significantly greater at 2 weeks of diabetes. When the urothelium was absent nerve-evoked contractions were reduced, but contractions remained significantly elevated at lower frequencies of stimulation ($<5\text{Hz}$) in diabetics. Interestingly, although relaxations of bladder strips to isoprenaline were unaltered by diabetes, removal of the urothelium unmasked significantly enhanced relaxations in strips from 2-week and 12-week diabetic animals. In conclusion, diabetes alters urothelial function. Enhanced urothelial ATP release may be involved in the hypercontractility observed at early time points of diabetes. These alterations are time-dependent and may contribute to the mechanisms at play during the development of diabetic bladder dysfunction.

Key words: ATP, urothelium, bladder dysfunction, diabetic complications, streptozotocin, diabetes mellitus

Introduction

Lower urinary tract complications are amongst the most common and costly complications of diabetes mellitus, and have been estimated to affect up to 87% of patients with diabetes¹, a rate greater than that of the widely known, and more widely researched complications, neuropathy and nephropathy². In particular, approximately 50% of patients diagnosed with diabetes are affected by diabetic bladder dysfunction³, a condition characterized by a wide range of clinical symptoms, from problems with storage through to voiding symptoms⁴. Patients may have impaired voiding reflexes, that result in increased residual volumes at the end of urination, increased bladder capacity, decreased bladder sensation and decreased rates of contraction of the bladder wall⁵. Alternatively, diabetic bladder dysfunction can also present as overactive bladder, with distressing storage symptoms including urinary urgency, frequency, nocturia and incontinence^{3, 4}. Thus, diabetic bladder dysfunction has a huge impact on patient quality of life and is a significant healthcare burden.

The pathogenesis of diabetic bladder dysfunction is not well understood. Studies have characterised both functional and structural changes within the bladder, including changes in bladder function and contractility⁶⁻¹¹, as well as alterations in bladder morphology, innervation and vasculature^{12, 13}. These changes are temporal in nature^{6, 7}, with the bladder undergoing a transition from a compensated state to a decompensated state with the duration and severity of diabetes⁶. However, alterations within the inner lining of the bladder, the urothelium, have been less well researched, particularly with relation to function.

The urothelium, whilst acting as a barrier to the urine, plays an essential role in signal transduction to the underlying nerves, smooth muscle and interstitial cells, influencing bladder function via the release of mediators including ATP and acetylcholine (ACh) during bladder

filling¹⁴. Diabetes has been shown to alter the bladder histologically, with progressive hypertrophy of the smooth muscle and urothelium with time¹⁰. Time-dependent breaches in the barrier structure of the urothelium, and upregulation of muscarinic and purinergic receptor expression, have also been reported¹⁵. This suggests that urothelial function may be altered in diabetes, although only a few studies have investigated this. These studies have demonstrated decreased release of prostaglandin E2 and F2 α from isolated urothelial layers¹⁶, and increased ATP release along with depressed nitric oxide release in bladders from 4-week diabetic rats¹⁷. Urine PGE2 has also been shown to be decreased in 12-week diabetic rats¹⁸. To date, there are no reports on the effects of diabetes on the release of the other key urothelial mediator, ACh. In addition, it is not clear whether alterations in ATP release vary with duration of diabetes. Thus, despite the growing evidence for the importance of the urothelium in bladder function, the impact of diabetes on urothelial function has not yet been fully elucidated. The aim of the study was to investigate urothelial mediator release from isolated whole bladders from streptozotocin-diabetic rats and the influence of the urothelium in modulating contractility of bladder strips.

Results

Effect of Diabetes on Animal Parameters

2-week diabetic animals lost body weight relative to age-matched controls ($P<0.01$), and 12 week diabetic animals gained significantly less weight ($P<0.001$) over the 12-week period compared to age-matched controls (Table 1). Bladder weights were significantly greater than age-matched controls at both time points (Table 1). Blood glucose levels greater than 30mM were displayed on the glucometer as 'high', and all diabetic animals had blood glucose levels greater than 30mM (Table 1).

Effect of Diabetes on Bladder Compliance

Isolated whole bladders were infused with saline to a volume of 1200 μ l, which would normally increase intraluminal pressure close to the threshold required for initiation of voiding. Bladders from 2-week diabetic animals were significantly more compliant than control bladders, and intraluminal pressure did not rise above 10mmHg during the filling period. The intraluminal pressure recorded following distension was significantly reduced in bladders from 2-week diabetic animals (2.52 ± 0.15 mmHg, $P<0.001$) compared to controls (35.60 ± 7.20 mmHg) (Fig. 1a), and in 12-week diabetic animals compared to controls (3.50 ± 0.93 mmHg vs 10.15 ± 0.83 , $P<0.001$) (Fig. 1b).

Effect of Diabetes on Intraluminal Mediator Release

Upon distension of whole bladders to a low volume (375 μ l), intraluminal ATP release was below the level of quantification. ATP release following distension to a high volume (1200 μ l) was however significantly greater ($P<0.05$) in bladders from 2-week diabetic animals compared to controls (Fig. 2a). At 12 weeks of diabetes intraluminal ATP release was similar to controls (Fig. 2a).

Intraluminal ACh was released following distension of bladders to both low and high volume. Diabetes of 2 and 12-weeks duration did not alter ACh release (Fig. 2b). ACh release was consistently less in samples collected following high volume distensions compared to low volume distensions (Fig. 2b).

Effects of Diabetes on Contractility of Isolated Bladder Strips

Electrical field stimulation evoked efferent-nerve mediated contractions of bladder strips, which were completely abolished by tetrodotoxin (1 μ M) (data not shown). Nerve-evoked contractions were significantly greater in 2-week diabetic animals versus controls (Fig. 3a), however there was no significant difference between 12-week diabetic and age-matched controls (Fig. 3b). Upon removal of the urothelium, nerve-evoked contractions remained significantly elevated in strips from 2-week diabetics only at the lower frequencies (<5Hz) (Fig. 3c). This effect was also observed in strips from 12-week diabetic animals (Fig. 3d).

Carbachol caused contractions of bladder strips, which were unaffected by diabetes (Fig. 4). Maximal contractions (2.21 ± 0.23 and 2.31 ± 0.41 mN/mg) and potency (pEC_{50} , negative log of the EC_{50}) of carbachol (5.84 ± 0.09 and 5.74 ± 0.08) were similar in strips from 2-week control and 2-week diabetics respectively. In strips from 12-week controls and diabetics, maximum responses and potency of carbachol were also similar (1.16 ± 0.13 and 0.92 ± 0.17 mN/mg, pEC_{50} 6.28 ± 0.09 and 6.22 ± 0.09 respectively). Removal of the urothelium did not affect carbachol-induced contractions in any of the animal groups (Fig. 4).

Addition of KCl produced a pre-contraction of bladder strips, which was similar in control and diabetics (2.08 ± 0.34 vs 2.33 ± 0.61 mN/mg, 2-week control vs diabetics; 1.13 ± 0.23 vs 1.05 ± 0.26

mN/mg 12-week control vs diabetics), and unaffected by removal of the urothelium (2.50 ± 0.27 vs 2.15 ± 0.46 mN/mg, 2-week control vs diabetics without urothelium; 1.08 ± 0.26 vs 1.15 ± 0.49 mN/mg 12-week control vs diabetics without urothelium). Isoprenaline caused relaxation of the KCl-pre-contracted bladder strips. Maximal relaxations to isoprenaline were similar at 2 weeks (-0.37 ± 0.05 mN/mg in diabetics compared to controls -0.36 ± 0.06 mN/mg). However, when the urothelium was removed isoprenaline relaxations were significantly greater in strips from 2-week diabetics compared to controls (-0.65 ± 0.19 vs -0.29 ± 0.07 mN/mg, $P < 0.05$) (Fig. 5a). pEC_{50} values for isoprenaline were similar at 2 weeks (6.73 ± 0.49 vs 5.95 ± 0.28 , diabetics vs controls) and removal of the urothelium did not affect these (6.73 ± 0.49 vs 6.68 ± 0.47 , with vs without urothelium). Maximal responses and pEC_{50} values for isoprenaline were also similar in 12-week controls and diabetics (-0.22 ± 0.04 vs -0.16 ± 0.01 mN/mg, pEC_{50} 6.35 ± 0.17 vs 6.35 ± 0.17). However, removal of the urothelium from bladder strips from 12-week diabetic animals unmasked significantly greater relaxations to isoprenaline (-0.41 ± 0.07 mN/mg, $P < 0.05$) when compared to intact 12-week diabetic strips (-0.16 ± 0.01 mN/mg), and an increase in potency (6.96 ± 0.21 , $P < 0.05$) (Fig. 5c).

Discussion

The aim of the present study was specifically to investigate urothelial mediator release from isolated whole bladders, and the influence of the urothelium in modulating contractility of bladder strips from the streptozotocin-diabetic rat. The urothelium plays an important role in transmission of information from the bladder wall to the nervous system, and is a major source of non-neuronal ATP¹⁴. ATP released from urothelial cells as the bladder fills and stretches activates sensory nerves, via purinergic P2X3 receptors, leading to increased afferent signalling to the CNS, and initiating micturition and bladder emptying¹⁹. Increased urothelial ATP release has been associated with a number of bladder disorders, and is seen in bladder strips from patients with overactive bladder²⁰ and painful bladder syndrome²¹, as well as in urothelial cells from patients with interstitial cystitis²². ATP is thought to sensitise afferent nerves and interstitial cells and alter bladder activity. In the present study, ATP release into the bladder lumen was measured following distension with saline, to mimic bladder filling. Both low and high volume distensions were examined, to determine whether ATP release was altered with the degree of distension in diabetes. The volume of saline infused during high volume distension produced an intraluminal pressure of around 25mmHg in control bladders, which is close to that which would normally initiate voiding²³. ATP released into the lumen following low volume distension was below the level of detection. However, following high volume distension ATP was quantifiable in all samples, suggesting that the degree of distension influences the amount of ATP released. The relationship between distension of the bladder and ATP release is not yet clear, although stretch-related ATP release has been widely reported in bladder strips^{20, 24, 25}, and ATP increases in a pressure-dependent manner in the bladder in a number of species²⁶⁻²⁹. The degree of stretch at low volume distensions in the present study may not have been sufficient to activate stretch-dependent ATP release. Although in patients ATP release is important for sensation in the early stages of bladder filling³⁰. An alternative

explanation may be that small amounts of ATP released at low volume distensions are undetectable due to the activity of ecto-ATPase enzymes, which are known to be expressed within the urothelium³¹.

Despite this complexity at low volume bladder distensions, ATP release following high volume distension was enhanced by diabetes of 2-weeks. Similarly, ATP release has been shown to be enhanced at 4 weeks of diabetes¹⁷. Thus, increased ATP release may be a feature of the early stages of diabetic bladder dysfunction. In other types of bladder dysfunction increased ATP release is thought to contribute to aberrant sensation and lead to altered bladder activity^{17, 32}. In the present study, ATP release was not significantly greater at 12-weeks of diabetes compared to age-matched controls. This suggests a transition of urothelial function from a compensated state at 2 weeks to a decompensated state at 12 weeks, as demonstrated for a number of other parameters of bladder function in diabetes^{6, 7, 33}. Alternatively, rather than a temporal change in ATP release with duration of diabetes, it may be that ATP metabolism is affected. Whilst, to our knowledge there have been no reports of altered ecto-ATPase activity within the bladder in diabetes, activity of these enzymes is altered in bladder tissues from patients with overactive bladder³⁴. Regardless of the underlying mechanism, here we demonstrate temporal changes in intraluminal ATP in diabetic bladder dysfunction, which has not been reported previously.

The urothelium also synthesises and releases the mediator ACh³⁵. Whilst less well understood than ATP, ACh may act on the underlying cell types, playing a role in bladder sensation and contraction¹⁴. ACh also acts in an autocrine and paracrine manner, to stimulate release of other mediators including NO and ATP^{14, 35}. In the present study ACh release was detected in intraluminal samples following both low and high volume distensions, although release was not affected by diabetes. ACh release was consistently lower following stretch to high volume

distension when compared to low volume distension. Non-neuronal ACh release from urothelial cells and human bladder strips increases with mechanical stretch^{35, 36}, and so the most likely explanation for these findings is that during the longer filling time associated with the high volume distension ACh was broken down by endogenous acetylcholinesterases, known to be present in the bladder mucosa³⁷.

During bladder filling the volume infused during high distension was sufficient to increase intraluminal pressure beyond 25mmHg in control animals, which is close to the threshold at which normal voiding would occur²³. However, diabetes resulted in highly compliant bladders, in which pressure increased very little in response to the high volume distension. This is consistent with previous *in vivo* cystometry studies, in which large increases in volume were needed for diabetic animals to sense a full bladder and to trigger micturition³⁸. Other studies have also shown an increase in bladder weight, bladder capacity and *in vivo* compliance in diabetes^{12, 39}. Interestingly, at 12 weeks there were also age-related changes in bladders from control animals, which showed similar high compliance with very little increase in pressure in response to the high volume distension. It is well known that bladders undergo remodelling with age, with a gradual loss of smooth muscle and increase in collagen, a larger filling capacity and functionally weaker smooth muscle and nerve-evoked contractions⁴⁰. Hence, the change in compliance at 12 weeks of diabetes may not reflect a diabetes-induced pathological alteration.

Locally, the urothelium can influence contractility of the underlying smooth muscle⁴¹, for example via the release of a urothelium-derived inhibitory factor (UDIF), found in the porcine and human bladder⁴²⁻⁴⁴, but as yet unidentified. Using urothelium-intact and denuded bladder strips we investigated whether diabetes alters this influence of the urothelium on bladder contractility. Diabetes did not affect contractile responses to the muscarinic receptor agonist

carbachol, consistent with several previous studies^{45, 46}, but in contrast to others showing depressed cholinergic contractions in 12-week diabetic bladder strips³⁷. In addition, removal of the urothelium did not affect contractions in control strips. This confirms that UDIF, which inhibits contractile responses to carbachol in pig and human bladders^{42, 44}, is not released from the rat bladder urothelium, as reported previously⁴⁷.

Relaxation responses of bladder strips to isoprenaline, a β -adrenoceptor agonist, were also unaltered by diabetes. However, removal of the urothelium unmasked greater relaxations in bladder strips from 2-week diabetic animals compared to denuded controls, and also enhanced relaxations in 12-week diabetic bladder strips relative to intact strips. These findings suggest that diabetes alters urothelium function in a complex manner. Both excitatory and inhibitory prostaglandins are released from the urothelium and are thought to influence detrusor activity⁴⁸, and increased levels of prostaglandins are considered biomarkers for lower urinary tract dysfunction⁴⁹. Release of PGE₂, an excitatory prostaglandin, which would oppose relaxation, was found to be reduced in urothelial preparations from diabetic rats¹⁶. Thus, whether our findings can be explained by the release of a different prostaglandin, or other excitatory substance, is not yet clear.

Immunohistochemical studies have demonstrated reduced bladder innervation¹³ and decreased cholinergic innervation⁵⁰ in diabetes. In the present study nerve-evoked contractions of bladder strips were enhanced by 2 weeks of diabetes, consistent with previous functional studies^{12, 51}. Interestingly, following removal of the urothelium, nerve-evoked responses of bladder strips from 2 weeks diabetic animals remained significantly enhanced only at lower frequencies of stimulation (below 5Hz). In addition, removal of the urothelium unmasked significantly greater contractions in 12-week diabetic strips, again at lower frequencies. Low frequency field

stimulation of bladder efferent nerves is thought to cause contractions mainly due to release of ATP, whilst cholinergic nerves are activated at higher frequencies^{12, 52}. Whilst we did not investigate atropine resistance of contractions at low frequencies of stimulation, enhanced purinergic-mediated nerve-evoked contractions, and atropine-resistance, have been widely demonstrated in a number of previous studies in both diabetic rat^{12, 53, 54} and rabbit⁵⁵ bladders, as well as in other types of bladder dysfunction^{12, 54}. Munoz *et al.* (2013) also showed that changes in the non-adrenergic, non-cholinergic NANC and purinergic components of nerve-evoked contractions is complex in diabetes, with plasticity in both NANC and P2X-mediated contractions, as well as muscarinic receptor-mediated alterations in NANC contractions via desensitisation mechanisms involving P2X2 receptors⁵⁶. The role of the urothelium in this complex plasticity of nerve-mediated contractions during the pathogenesis of diabetic dysfunction is not yet clear.

In conclusion, a large percentage of patients with diabetes mellitus will develop urinary complications and diabetic bladder dysfunction, with a significant impact on quality of life. Current research is focussed on understanding the pathogenesis of diabetic bladder dysfunction. This study provides evidence of time-dependent functional changes within the urothelium in diabetes, specifically with respect to urothelial ATP release and the influence of the urothelium on contractility.

Methods

Animal Model

All procedures were performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes and under approval of Griffith and Bond University Animal Ethics Committees.

Eight-week old male Wistar-Hannover rats were randomly allocated into four treatment groups: 2-weeks diabetic, 2-weeks controls, 12-week diabetic, and 12-week controls. Diabetes mellitus was induced via a single intraperitoneal injection of streptozotocin (65mg/kg, dissolved in 0.01M citrate buffer, pH 4.5). Control animals were age-matched to their respective diabetic group. Animals were maintained under environmentally controlled conditions, with 12-hour light-dark cycles and free access to food and water.

Animals were sacrificed via pentobarbital overdose (60mg/kg body weight, i.p.), 2 or 12 weeks following induction of diabetes. Bladders were removed, placed into iced Krebs bicarbonate solution (118.4mM sodium chloride, 24.9mM sodium bicarbonate, 4.7mM potassium chloride, 1.9mM calcium chloride, 1.15mM monopotassium phosphate and 11.7mM glucose), cleaned of connective tissue and whole wet bladder weight recorded. Blood was collected via cardiac puncture and blood glucose measured using an Accu-Check glucometer. Animals with blood glucose greater than 20mM were considered diabetic.

Isolated Whole Bladders: Measurement of Bladder Compliance and Intraluminal Mediator Release

Isolated whole bladders were cannulated with a two-way cannula via the urethra and secured with suture. The bladders were placed into modified tissue baths (5ml) containing gassed Krebs bicarbonate solution (95% O₂, 5% CO₂) heated to 37°C. The cannula was connected to a syringe pump and a pressure transducer, connected to a PC via a 1401 interface, using “Spike 2”

software version 3 (Cambridge Electronic Design, Cambridge, UK), to enable recording of intraluminal pressure during bladder filling and emptying. Following 30 minutes of equilibration, bladders were filled by infusion with isotonic saline (0.9% NaCl) at a rate of 150 μ l/min. Bladder distensions were performed by filling to either low volume (375 μ l) or high volume (1200 μ l). Following distension, the bladder was drained and intraluminal contents collected immediately on dry ice. These were stored at -30°C for later assay of the mediators ATP and ACh, as performed previously⁵⁷. Intraluminal ATP was measured using an ATP determination kit (Molecular Probes). This is a bioluminescence assay which determines ATP using luciferase and its substrate D-luciferin. The assay was performed according to manufacturer's instructions, whereby a standard curve using known concentrations of ATP was constructed and used to determine ATP in the intraluminal samples. Luminescence was measured using a microplate reader (Modulus, Promega). ACh was measured using an Amplex (®) Red acetylcholine assay (Molecular Probes), performed according to manufacturer's instructions. This is a fluorescence-based assay in which ACh in the samples is converted into choline by ACh, and the choline then oxidized by choline oxidase to betaine and hydrogen peroxide. Hydrogen peroxide reacts with Amplex Red reagent, in the presence of horseradish peroxidase, to generate the fluorescent product resorufin. ACh in the intraluminal samples determined using a standard curve of known concentrations of ACh. Fluorescence was measured on a microplate reader (Ex. 540/Em. 590 nm).

Contractility of Isolated Bladder Strips

Whole bladders were dissected into 3-4 longitudinal strips. Half of the strips were denuded of the urothelium by careful dissection under a microscope. Strips were mounted in EZ-Bath tissue baths (GlobalTown Microtechnology, FL, USA) filled with gassed Krebs bicarbonate solution (37°C, 95% O₂/5% CO₂), and allowed to equilibrate for 60 minutes under 150mN resting

tension, to allow examination of contractility. Changes in isometric developed tension were recorded using a Power Lab data acquisition system using 'LabChart[®]' software version 7.1.1 (AD instruments).

Cumulative-concentration response curves to the muscarinic agonist carbachol (Sigma-Aldrich) were performed to observe contractile responses. Following washout and return to baseline, relaxation responses to cumulative concentrations of the β -adrenoceptor agonist isoprenaline (Sigma-Aldrich) were obtained following precontraction with KCl (40mM). In separate bladder strips, nerve-evoked contractions to electrical field stimulation (EFS) via platinum electrodes were recorded. Frequency-response curves (0.2–20 Hz) were obtained using 5s trains of pulses (40 V, 0.01 ms pulse width) delivered every 100 s. Tetrodotoxin (1 μ M) (Sigma-Aldrich) was used to confirm that responses were neurogenic in nature and wet weight of bladder strips was recorded at the end of the experiments.

Statistical Analysis

GraphPad 'Prism' software (GraphPad, San Diego, CA, USA) was used to perform statistical analysis. For whole bladders, pressure-volume relationships were calculated, and data compared using unpaired two-way Student's *t*-tests. Intraluminal mediator release from isolated whole bladder preparations was expressed as mean \pm SEM of *n* experiments, where *n* was the number of bladders. Non-linear regression analysis of the concentration-response curves was used to determine the potency of the agonist (pEC₅₀, the log of the concentration of a drug that produces half of the maximal response). Mean (\pm SEM) pEC₅₀ values and maximum responses were calculated, whilst mean frequency-response curves were determined for nerve-evoked contractions. For contractile data *n* was the number of tissue strips. Data was normalised for tissue weight and compared using a one-way ANOVA with Tukey's post hoc test, or unpaired

two-tailed Student's t test where appropriate, as indicated. $P < 0.05$ was considered statistically significant.

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Figure Legends

Fig. 1. Bladder compliance

Ex vivo pressure-volume relationships of isolated whole bladders from 2-week (a) (n=11) and 12-week (b) (n=12) control and diabetic animals in response to distension, where bladders were filled at 150 μ l/min. (*P<0.05, **P<0.01, ***P<0.001 vs. respective control, Student's *t* test).

Fig. 2. Intraluminal ATP and ACh release from whole bladders

Intraluminal ATP release following distension of isolated whole bladders with high volume (1200 μ l) (a) and intraluminal ACh release following distension with low (375 μ l) and high volume (b) from 2-week and 12-week control and diabetic animals (n=5-7, *P<0.05, ***P<0.001, one-way ANOVA with Tukey). Note that ATP release was not measurable following distension with low volume.

Fig. 3. Nerve-evoked contractions of bladder strips

Frequency response curves of isolated bladder strips to electrical field stimulation (5s trains of pulses, 40 V, 0.01 ms pulse width, delivered every 100s) from 2-week (a & c) and 12-week (b & d) control and diabetic animals. Strips are intact (a & b) or denuded of urothelium (c & d). (n=6-8, *P<0.05 vs respective control, Student's *t* test).

Fig. 4. Contractility of bladder strips to carbachol

Cumulative concentration-response curves showing contractions of isolated bladder strips to carbachol from 2-week (a) and 12-week (b) controls and diabetics. Strips are intact or denuded of urothelium. Data are expressed as mN/mg tissue weight (n=5-8).

Fig. 5. Relaxation responses of bladder strips to isoprenaline

Cumulative relaxation-response curves to isoprenaline in isolated bladder strips from 2-week denuded control and diabetic (a), 2- versus 12-week controls (b) and 12-week intact and denuded diabetic animals (c). Data are expressed as mN/mg tissue weight. (n=5-8, *P<0.05 denuded diabetic vs denuded control, **P<0.001 denuded diabetic vs intact diabetic, one-way ANOVA with Tukey).